

AD\_\_\_\_\_

Award Number: W81XH-05-1-0497

TITLE: Adipose Estrogen and Increased Breast Cancer Risk in Obesity: Regulation by Leptin and Insulin

PRINCIPAL INVESTIGATOR: Dr. Fahumiya Samad

CONTRACTING ORGANIZATION: La Jolla Institute for Molecular Medicine  
San Diego, CA 92121

REPORT DATE: September 7, 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE  |             |                         |                            | Form Approved<br>OMB No. 0704-0188                       |   |
|--|-------------|-------------------------|----------------------------|--|---|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>  |             |                         |                            |  |   |
| 1. REPORT DATE (DD-MM-YYYY)<br>10-09-2007  |             | 2. REPORT TYPE<br>Final |                            | 3. DATES COVERED (From - To)<br>Aug 15, 2005-Sep 7, 2007 |   |
| 4. TITLE AND SUBTITLE<br><br>Adipose Estrogen and Increased Breast Cancer Risk in Obesity: Regulation by Leptin and Insulin  |             |                         |                            | 5a. CONTRACT NUMBER                                      |   |
|  |             |                         |                            | 5b. GRANT NUMBER<br>W81XWH-05-1-0497                     |   |
|  |             |                         |                            | 5c. PROGRAM ELEMENT NUMBER                               |   |
| 6. AUTHOR(S)<br>Dr. Fahumiya Samad<br><br>E-Mail: fsamad@tpims.org   |             |                         |                            | 5d. PROJECT NUMBER                                       |   |
|  |             |                         |                            | 5e. TASK NUMBER  |   |
|  |             |                         |                            | 5f. WORK UNIT NUMBER                                     |   |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>La Jolla Institute for Molecular Medicine<br>San Diego, CA 92121   |             |                         |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER                 |   |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012  |             |                         |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)                         |   |
|  |             |                         |                            | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)                   |   |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br>Approved for Public Release; Distribution Unlimited   |             |                         |                            |  |   |
| 13. SUPPLEMENTARY NOTES  |             |                         |                            |  |   |
| 14. ABSTRACT Clinical studies suggest that obesity increases the risk for breast cancer and there is convincing evidence that post-menopausal breast cancer risk is highly correlated with serum estrogen levels. One potential link between obesity and breast cancer risk is increased estrogen production by the adipose tissue itself. The adipose tissue produces the enzyme aromatase which catalyses the biosynthesis of estrogen from androgen and also 17-beta-hydroxysteroid dehydrogenase (17-beta HSD) important for the conversion of estrone to estradiol. Our studies have identified two key molecules (insulin and leptin) in obesity that regulates aromatase and 17-beta HSD synthesis in adipose tissues and in adipocytes. The identification of these target molecules that may ultimately induce estrogen production in the setting of obesity may provide a unique therapeutic preventive strategy to reduce systemic estrogen levels and thereby reduce post-menopausal breast cancer risk associated with obesity. |             |                         |                            |  |   |
| 15. SUBJECT TERMS<br>Obesity, breast cancer, insulin, leptin, estrogen, aromatase, 17•HSD  |             |                         |                            |  |   |
| 16. SECURITY CLASSIFICATION OF:  |             |                         | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES                                      | 19a. NAME OF RESPONSIBLE PERSON           |
| a. REPORT  | b. ABSTRACT | c. THIS PAGE            |                            |  | USAMRMC                                   |
| U  | U           | U                       | UU                         | 9  | 19b. TELEPHONE NUMBER (include area code) |

## Table of Contents

|                                   | <u>Page</u> |
|-----------------------------------|-------------|
| Introduction.....                 | 4           |
| Body.....                         | 4-8         |
| Key Research Accomplishments..... | 9           |
| Reportable Outcomes.....          | 9           |
| Conclusion.....                   | 9           |
| References.....                   | 9           |
| Appendices.....                   | N/A         |

## Introduction:

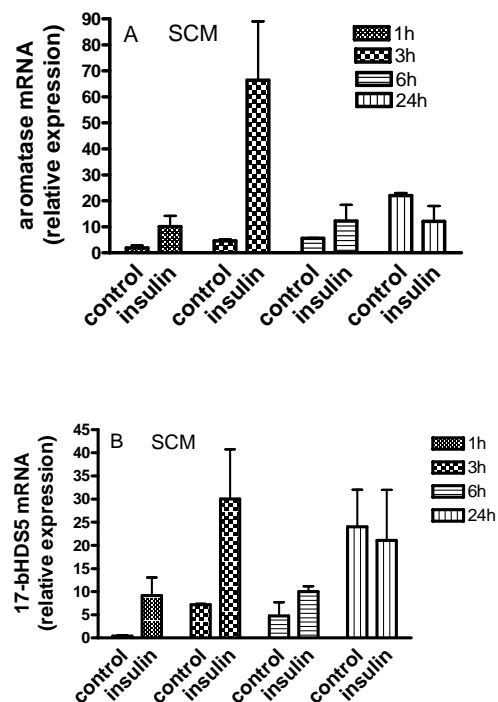
Clinical studies suggest and obesity increases the risk for breast cancer and there is convincing evidence that post-menopausal breast cancer risk is highly correlated with serum estrogen levels. One potential link between obesity and breast cancer risk is increased estrogen production by the adipose tissue itself. The adipose tissue produces the enzyme aromatase which catalyses the biosynthesis of estrogen from androgen and also 17- $\beta$ -hydroxysteroid dehydrogenase (17- $\beta$ HSD) important for the conversion of estrone to estradiol. In spite of this the mechanisms regulating the adipose expression of aromatase and 17- $\beta$ HSD is however currently unknown. Identifying the mediators in obesity that regulate aromatase and 17- $\beta$ HSD synthesis in adipose tissues and in adipocytes may provide a unique therapeutic preventive strategy to reduce systemic estrogen levels and thereby reduce post-menopausal breast cancer risk associated with obesity.

## BODY:

### Task 1 : Perform in vitro studies on the regulation of Aromatase and 17- $\beta$ HSD (types 4, 5) synthesis in murine and human adipocytes in response to insulin and leptin

In order to determine the regulation of Aromatase and 17- $\beta$ HSD in adipocytes we initially standardized an in vitro murine adipocyte cell culture system, In this model, 3T3, L1 pre adipocytes were grown and differentiated into adipocytes after a brief exposure of confluent pre-adipocytes to insulin and dexamethasone. This treatment triggered the differentiation of pre adipocytes to fully differentiated

lipid filled adipocytes over the course of 2-3 weeks. Fully differentiated 3T3-L1 adipocytes were treated with insulin (100nM), and cells harvested at various times after treatment for total RNA extraction. Aromatase and 17- $\beta$ HSD5 mRNA expression was determined by real time RT-PCR. Treatment of fully differentiated 3T3-L1 adipocytes with 100nM insulin in serum containing media (SCM) significantly induced both Aromatase (Fig 1 A) and 17- $\beta$ HSD5 mRNA (Fig 1 B) expression in these cells. Aromatase mRNA expression was induced significantly as early as 1 hour after insulin treatment and this expression continued to increase by 3 h after insulin treatment. Similar kinetics of induction of mRNA was also observed after insulin treatment for the expression of 17- $\beta$ HSD5 mRNA (Fig. 1B). 17- $\beta$ HSD5 mRNA expression was dramatically induced at 1 and 3 h after insulin treatment. These studies suggest that hyperinsulinemia associated with obesity may contribute to the increased expression of Aromatase and 17- $\beta$ HSD5 mRNA from adipocytes.



**Fig. 1:** Aromatase and 17- $\beta$  HSD5 mRNA expression in response to insulin in cultured 3T3-L1 adipocytes. For all conditions n=6±SD

In a different set of experiments fully differentiated 3T3-L1 adipocytes were treated with leptin (100nM) and the kinetics of both Aromatase and 17- $\beta$ HSD5 mRNA regulation determined at 3, 6, and 24 hrs after leptin treatment. Aromatase mRNA expression was significantly induced in adipocytes at 3 and 6 hrs after leptin treatment (Fig. 2A). In contrast to Aromatase mRNA expression, the gene expression of  $\beta$ HSD5 was reduced 3 hrs after leptin treatment but was significantly increased after exposure to leptin for 24 hrs. These data suggest that long term chronic exposure to leptin may increase the expression of  $\beta$ HSD5 from adipocytes.

Since human obesity is associated with elevated levels of leptin, our data suggest that hyperleptinemia associated with obesity may contribute not only to Aromatase gene expression but also to increased levels of  $\beta$ HSD5 in adipocytes. These results thus support our primary hypothesis that the hyperleptinemia and hyperinsulinemia associated with obesity may induce the expression of Aromatase and  $\beta$ HSD5 from the adipose tissue, specifically from adipocytes. Studies to determine whether the increase in Aromatase and  $\beta$ HSD5 gene expression in response to insulin and leptin actually leads to increased estrogen secretion into the conditioned media are ongoing.

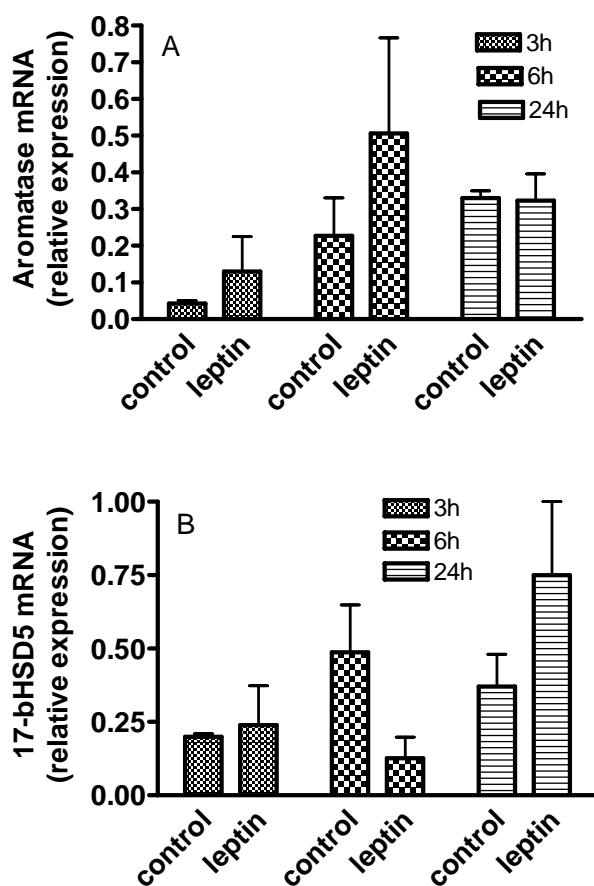


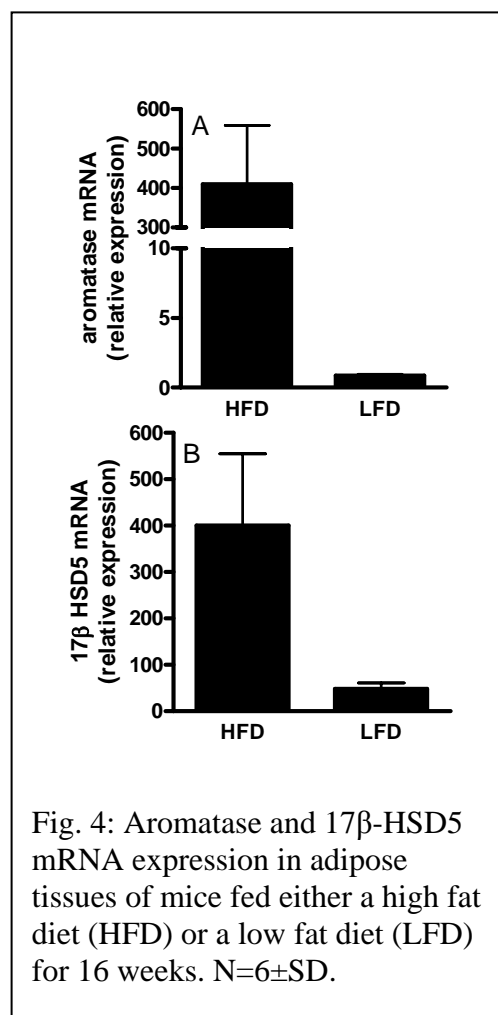
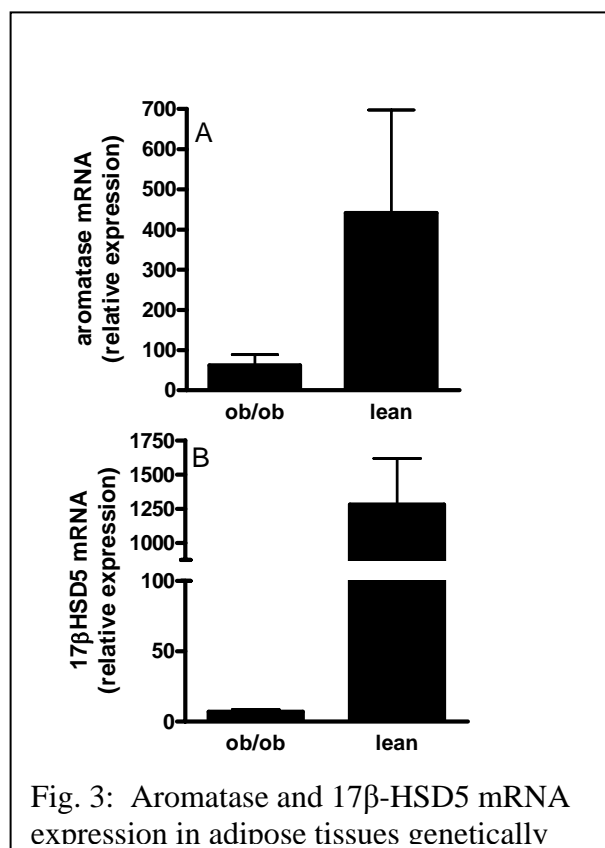
Fig. 2: Aromatase and 17- $\beta$  HSD5 mRNA expression in response to leptin in cultured 3T3-L1 adipocytes. For all conditions n=6 $\pm$ SD

Since obesity is usually associated with insulin resistance we had also proposed to determine the induction of Aromatase and  $\beta$ HSD5 mRNA expression in response to insulin and leptin in insulin resistant adipocytes. 3T3-L1 adipocytes were treated with low doses (2ng/ml) of tumor necrosis factor  $\alpha$  consecutively for 3 days. Metabolic insulin resistance was determined by measuring insulin mediated glucose uptake. Glucose uptake was reduced by 50-70% in adipocytes treated with TNF- $\alpha$  compared to untreated cells, suggesting that glucose uptake was blunted in these cells. Thus, we have been able to standardize conditions in our cell culture system to mimic metabolic insulin resistance. These “insulin-resistant” adipocytes are currently being used to determine insulin and leptin mediated regulation of Aromatase and  $\beta$ HSD5 expression by these cells. Preliminary studies indicate that these “metabolically insulin resistant” adipocytes remain sensitive to insulin in terms of aromatase and 17 $\beta$  HSD5 induction. These results suggests that the signaling pathways by which insulin induces aromatase and 17 $\beta$ HSD5 expression do not become “insulin resistant” but continue to respond to the hyperinsulinemia associated with

obesity.

**Task 2: Perform in vivo studies on the regulation of Aromatase and 17-beta HSD (types 4, 5) expression in response to leptin and/or insulin using lean, diet-induced obese and genetically obese mice.**

While our hypothesis was that increased insulin and leptin levels associated with obesity may drive the up regulation of both aromatase and 17- $\beta$ HSD expression in adipose tissues, we had not in fact directly compared the expression of these genes in adipose tissues of lean and obese mice. We used both the genetically obese ob/ob mice, and a diet induced model of obesity in normal C57BL/6J mice to determine aromatase and 17- $\beta$ HSD expression in adipose tissues. In the model of diet induced obesity lean C57BL/6J mice were placed either on a high fat diet (HFD; 60% kcal from fat) or a low fat diet (10% kcal from fat) for 16 weeks. On the HFD the mice became obese, insulin resistant and diabetic. The genetically obese ob/ob mice is a severe model of obesity and these mice also lacks the satiety hormone leptin. The diet induced model of obesity is a milder and a more physiologically relevant form of obesity which better mimics the human condition. Moreover, in contrast to the genetic model, the HFD-induced obese mice also show increased levels of leptin which is similar to human obesity. In the genetically obese (ob/ob) mice, the expression levels of both aromatase and 17 $\beta$ -HSD were in fact reduced in ob/ob mice when compared to its lean counterparts (Fig. 3). However, when we determined the levels of these genes in the adipose tissues of diet-induced obese mice there was a significant increase in both the aromatase and 17 $\beta$ -HSD expression in mice fed the HFD compared to those on the LFD (Fig. 4). Based on the results we obtained in these two models of obesity, we hypothesized that the decrease in the expression of aromatase and 17 $\beta$ -HSD5 in adipose tissues of the



ob/ob mice is probably due to the lack of leptin, and, that increased leptin associated with obesity such as in the diet induced model of obesity may actually lead to elevated levels of adipose aromatase and

17- $\beta$ HSD5 expression. We tested this hypothesis by injecting ob/ob mice with exogenous leptin and measured the expression of aromatase and 17 $\beta$ -HSD5 in the adipose tissue 3 hours after treatment. For these experiments, groups (n=6) of ob/ob mice were injected with leptin (10 $\mu$ g/mouse). 3 hours later, mice were sacrificed and blood and adipose tissues were harvested. Total RNA was extracted from adipose tissues and the expression of Aromatase and 17- $\beta$ HSD4 mRNA expression determined by real time RT-PCR. As indicated in Figure 5, leptin treatment of the leptin deficient ob/ob mice led to a dramatic increase in the expression of both aromatase and 17 $\beta$ -HSD5 mRNA levels in the adipose tissues. Leptin treatment of lean mice similarly induced the adipose expression of aromatase 7- $\beta$ HSD4 mRNA (Fig. 6, 7).

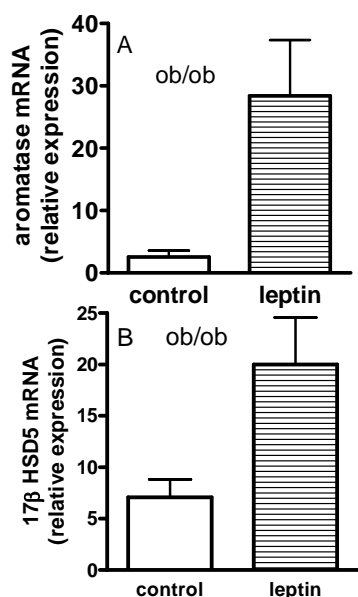


Fig. 5: Aromatase and 17 $\beta$ -HSD5 mRNA expression in adipose tissues of genetically obese ob/ob treated with leptin. N=6 $\pm$ SD.

These studies support our hypothesis that hyperleptinemia associated with obesity leads to the up regulation of aromatase and 17 $\beta$ -HSD5 mRNA in adipose tissues.

We next performed experiments to determine whether insulin induces the expression of Aromatase and 17- $\beta$ HSD expression in adipose tissues in vivo. Groups (n=6) of C57BL/6J lean mice were injected with insulin (humulin, 5 IU), and 3 hours later, mice were sacrificed and blood and adipose tissues harvested. Total RNA was extracted from adipose tissues and the expression of Aromatase and 17- $\beta$ HSD mRNA expression determined by real time RT-PCR. As shown in Fig 8, insulin treatment also induced a dramatic and significant expression of aromatase mRNA in adipose tissue of lean mice. Our preliminary studies had previously shown that Aromatase gene expression is also induced by insulin in the obese, ob/ob mice that are insulin resistant. Studies are ongoing to confirm and extend the results relating to the insulin induction of aromatase gene expression in adipose tissues from insulin resistant genetic and diet-induced obese mice.

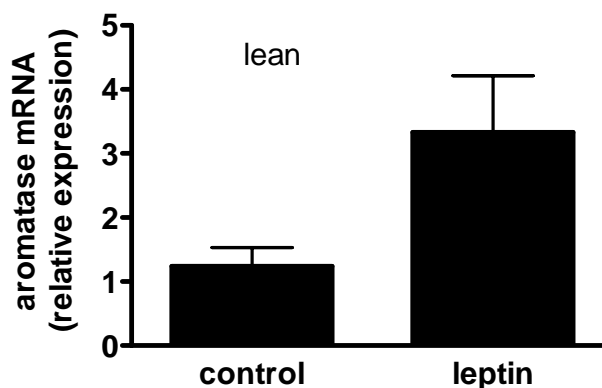


Fig 6: Aromatase mRNA expression in adipose tissues from insulin treated C57/BL6 lean mice. N=6 $\pm$ SD

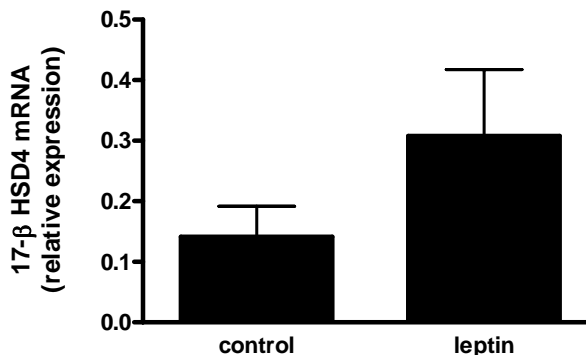
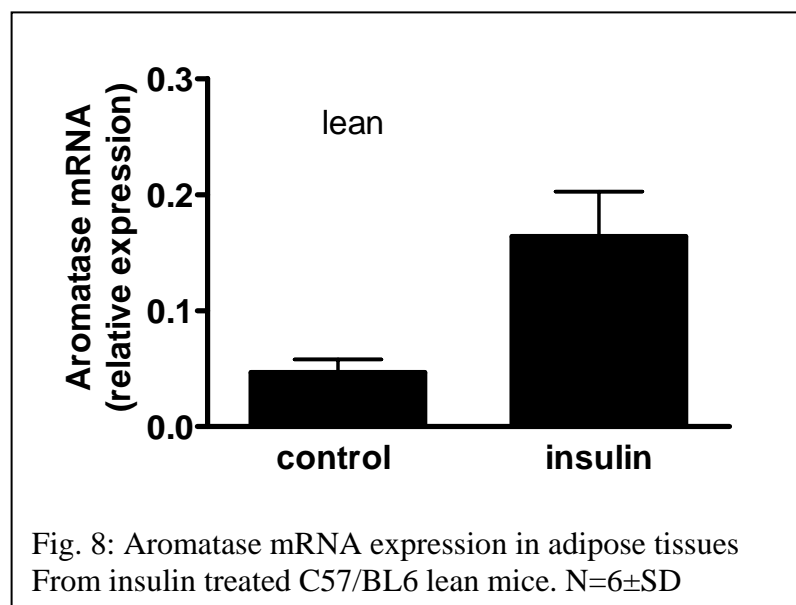


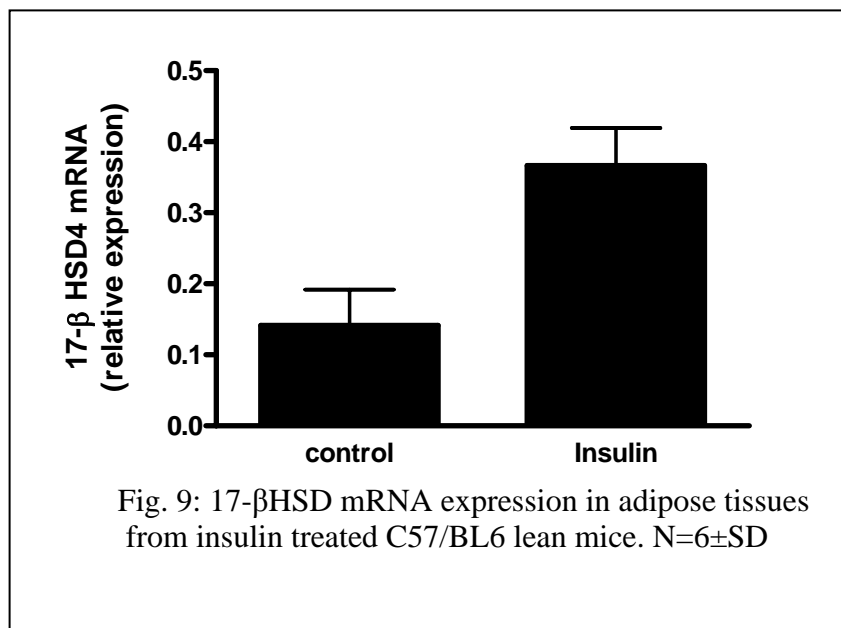
Fig 7: 17- $\beta$ HSD mRNA expression in adipose tissues from insulin treated C57/BL6 lean mice. N=6 $\pm$ SD

We next determined the expression of 17- $\beta$ HSD mRNA in adipose tissues of insulin treated C57BL/6J lean mice. Interestingly, the expression of 17- $\beta$ HSD5 mRNA levels was very low in the adipose tissues of these mice and hence we were unable to make any meaningful conclusions in relation to its



genetic and diet-induced obese mice.

expression. However, we show that 17- $\beta$ HSD4 mRNA was expressed in adipose tissues of control untreated C57BL.6J mice, and its expression was significantly and dramatically induced 3 hr after insulin treatment (Fig. 9). These data suggest that the hyperinsulinemia associated with insulin resistance and obesity may drive the expression of both Aromatase and 17- $\beta$ HSD mRNA and thereby contribute to increased estrogen secretion from adipose tissues in obesity. Studies are ongoing to confirm and extend the results relating to the insulin induction of 17- $\beta$ HSD gene expression in adipose tissues from insulin resistant



Together, our in vivo data suggest that increased elevated levels of insulin and leptin, associated with obesity may increase breast cancer risk by inducing the adipose expression of both Aromatase and 17- $\beta$ HSD. While Aromatase catalyses the biosynthesis of estrogens from androgens, 17- $\beta$ HSDs are important for the conversion of estrone to estradiol (1). Thus the increased expression of both of these enzymes is important for the production of biologically active estrogen. Are data suggest that insulin and leptin are important in this process.

## Key research Accomplishments:

- Aromatase and 17- $\beta$  HSD5 mRNA expression is increased in the adipose tissues of genetically obese ob/ob mice that are leptin deficient compared to its lean counterpart.
- Aromatase and 17- $\beta$  HSD5 mRNA levels are increased in the adipose tissues of mice fed a high fat diet compared to those on a low fat diet.
- Aromatase and 17- $\beta$ HSD4 gene expression is increased in vivo in adipose tissues of ob/ob and C57BL/6J lean mice treated with **leptin**
- Aromatase and 17- $\beta$  HSD5 mRNA expression is also increased in response to **leptin** in cultured 3T3-L1 adipocytes.
- Aromatase and 17- $\beta$ HSD4 gene expression is increased in vivo in adipose tissues of C57BL/6J lean mice treated with **insulin**
- Aromatase and 17- $\beta$  HSD5 mRNA expression is also increased in response to insulin in cultured 3T3-L1 adipocytes.

The results of our studies have identified two key mediators in obesity (insulin and leptin) that regulates aromatase and 17- $\beta$ HSD synthesis in the adipose tissue and in adipocytes. The results from this study may provide a unique therapeutic prevention strategy to reduce systemic estrogen levels and thereby reduce postmenopausal breast cancer risk associated with obesity.

**Reportable outcomes:** Manuscript in preparation.

## Conclusion:

In conclusion, our studies support the hypothesis that hyperleptinemia and hyperinsulinemia associated with obesity can induce not only aromatase but also 17- $\beta$  HSD synthesis from the adipose tissue which may lead to an increase secretion of estrogen from an expanded adipose tissue in obesity. These studies provide a potential molecular link by which obesity leads to increased risk for breast cancer. Targeting the adipose production of enzymes leading to elevated estrogen production may provide a feasible therapeutic option to reduce breast cancer risk.

## References:

1. Calle, E.E. and Kaaks, R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nature Reviews* 4:579-588, 2004
2. Harvie, M., Hooper, L. and Howell, A.H. Central obesity and breast cancer risk. *Obesity Rev.* 4: 157-173, 2003
3. Stephenson, G.D., and Rose, D.P. Breast Cancer and Obesity: An Update. *Nutrition and Cancer.*45(1):1-6, 2003
4. Trayhurn, P. and Beattie, J.H. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc. Of the Nutr. Soc.* 60(3):329-339, 2001
5. Tessitore, L. et al. adipocyte expression and circulating levels of leptin increase in both gynecological and breast cancer patients. *Intern. J. Oncology.* 24:1529-1535, 2004.